Capsule stiffness regulates the efficiency of pancreatic differentiation of human embryonic stem cells

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Abstract

Encapsulation of donor islets using a hydrogel material is a well-studied strategy for islet transplantation, which protects donor islets from the host immune response. Replacement of donor islets by human embryonic stem cell (hESC) derived islets will also require a means of immune-isolating hESCs by encapsulation. However, a critical consideration of hESC differentiation is the effect of surrounding biophysical environment, in this case capsule biophysical properties, on differentiation. The objective of this study, thus, was to evaluate the effect of capsule properties on growth, viability, and differentiation of encapsulated hESCs throughout pancreatic induction. It was observed that even in the presence of soluble chemical cues for pancreatic induction, substrate properties can significantly modulate pancreatic differentiation, hence necessitating careful tuning of capsule properties. Capsules in the range of 4-7 kPa supported cell growth and viability, whereas capsules of higher stiffness suppressed cell growth. While an increase in capsule stiffness enhanced differentiation at the intermediate definitive endoderm (DE) stage, increased stiffness strongly suppressed pancreatic progenitor (PP) induction. Signaling pathway analysis indicated an increase in pSMAD/pAKT levels with substrate stiffness likely the cause of enhancement of DE differentiation. In contrast, sonic hedgehog inhibition was more efficient under softer gel conditions, which is necessary for successful PP differentiation.

Key Words: human embryonic stem cells; Pancreatic Differentiation; substrate stiffness; diabetes; Alginate Encapsulation

1.0. Introduction

Type 1 diabetes (T1D) is an autoimmune disease affecting millions of people worldwide, wherein the immune system destroys the insulin producing β cells within the Islets of Langerhans [1]. While transplantation of donor islets has been demonstrated as a viable therapy by the Edmonton protocol (REF), donor islets are scarce and a high number is required to overcome hypoxia-induced cell death [2]. Alternately, β cells can be derived from human pluripotent stem cells (hPSCs), which have the capacity to self-renew and differentiate, making them attractive for regenerative medicine and cell therapy applications. A significant amount of work has focused on deriving β – like cells from pluripotent stem cells, including work done in our own lab [3-6]. Recently, the Melton group and the Kieffer group have reported a significant breakthrough in generating glucose responsive insulin producing β cells from hPSCs *in vitro* [3, 7]. While this will overcome the cell source restriction, the next step towards successful clinical translation of hPSC derived β cells for T1D therapy will require protection of the cells from the host immune system [8].

A well-studied approach to immunoisolate implanted primary islets for T1D treatment is to encapsulate the donor islets within a material which acts as a semipermeable membrane. This allows for the diffusion of nutrients and waste, but isolates and protects the cells from the larger immune cells [9-12]. Alginate is well-suited for this purpose because cells can be encapsulated with high viability, while having excellent biocompatibility and providing immunoisolation of the encapsulated cells. Recently, Omid *et al.* have shown that barium alginate capsulesHence it will be relevant to evaluate if alginate capsules can also be used to deliver hPSC-derived islet-like cells. Due to its excellent biocompatibility, alginate has already been tested for encapsulation and differentiation of mouse [13-16] and human [17-22] pluripotent stem cells (m/h-PSCs). In our recent work, we have reported successful derivation of insulin producing β -cells from hPSCs

encapsulated in calcium alginate capsules, towards a directly transplantable T1D treatment option [23]. Interestingly, we even observed significant enhancement in pancreatic maturation of hPSCs when differentiated under encapsulation, which renders calcium alginate capsules as an attractive platform for differentiation, in addition to immune protection.

While calcium alginate has been extensively used in hPSC literature, it is limited in its *in-vivo* applications because calcium ions can be easily displaced by monovalent cations, such as sodium, resulting in weakening of the capsules over time [24, 25]. Alginate is composed of mannuronic (M) and guluronic (G) acid, which forms a 3D network when divalent cations bind with the G residues of two adjacent polymer chains [26]. While calcium is commonly used for cell encapsulation, other divalent cations such strontium and barium can also be used. In comparison to calcium, barium binds to alginate with a much higher affinity, resulting in more robust capsules [27, 28]. Thus barium alginate (BAIg) capsules have been extensively used for encapsulation of islets for T1D treatment [29-33]. However, BAIg has been less explored for pluripotent stem cell encapsulation, with few reported studies in adult stem cells [34-37]. The only successful study with hESCs is by Dean *et al.*, who showed that hESCs encapsulated in BAIg could survive and differentiate when transplanted in mice for a period of four weeks [38]. Thus, the favorable mechanical properties of BAIg capsules warrant investigation of its potential for hESC encapsulation and differentiation.

The effect of insoluble physical cues, such as substrate stiffness or extracellular matrix (ECM) molecules, on stem cell differentiation is well-established [39-42]. In the context of endoderm specific differentiation, our group has demonstrated the feasibility of driving early germ layer differentiation of mESCs by modifying the properties alginate and fibrin substrates, in the absence of chemical inducers [43-46]. Hence even though pancreatic differentiation of encapsulated hESCs will be directed by chemical cues, it is likely that capsule properties will

affect the fate of encapsulated cells. Information is largely lacking in the context of pancreatic differentiation, and is the primary objective of this study. The physical properties of alginate capsules can be modulated by changing the alginate (M/G ratio) and/or cation (Ca, Ba, Sr) type and concentration [28, 47]. As a general rule of thumb, increasing cation concentration will increase the stiffness of the resulting capsule by higher crosslinking of G residues; these effects are further enhanced by cations such as barium which have higher binding affinity [28, 48]. All of these can affect the fate and response of encapsulated cells [49-51]. Hence, while engineering an encapsulation system for hPSCs, it is necessary to evaluate the effect of substrate properties on the fate of encapsulated cells.

The objective of this study was to investigate, for the first time, the use of BAAlg encapsulation for pancreatic differentiation of hESCs, and determine how BAAlg properties influence growth and subsequent differentiation of encapsulated hESCs. Overall it was observed that the efficiency of chemical induction was largely dependent on the properties of encapsulating substrate, even though diffusion was never restrictive within the capsules. Cell growth was observed to be favorable under the low stiffness regime, and was highly suppressed under high stiffness conditions. Interestingly, the effect of differentiation was more complex and differed based on stage of differentiation, possibly due to the complexity of the interaction of physical cues with non-linear signaling pathways. Increased alginate capsule stiffness appeared to promote TGF β signaling during the definitive endoderm (DE) stage, which enhanced DE differentiation. However, increased substrate stiffness also promoted sonic hedgehog signaling at the pancreatic progenitor (PP) stage, which suppressed PP differentiation. Overall, cell growth and hESC-PP differentiation was found to be favorable in the stiffness range of approximately 4-7 kPa.

2.0. Materials and Methods

2.1. Human embryonic stem cell culture

Undifferentiated (UD) H1 hESCs (WiCell) were maintained on hESC-qualified Matrigel (BD Biosciences) coated tissue culture plastic for 5-7 days in mTeSR1 (StemCell Technologies) at 37°C and 5% CO₂ before passaging. Experiments were performed with p55-p85 hESCs.

2.2. Barium alginate encapsulation of hESCs

A single cell suspension of UD hESCs was encapsulated by modifying our previous encapsulation protocol with the use of BaCl₂ [23] (details in SI methods). hESCs were encapsulated using 10, 15, 20, 50 or 100 mM BaCl₂. Alginate capsules were incubated for 6-8 min in the BaCl₂ solution. Capsules were washed three times with DMEM/F12 and suspended in mTeSR1 with 10 µM Y-27632 for 4 day, followed by 2 days in mTeSR1 alone to allow for colony formation, prior to differentiation.

2.3 Barium alginate capsule characterization

Alginate disks were formed using a 1.7 cm diameter mold, and crosslinked with 10, 15, 20, 50, and 100 mM BaCl₂. AFM force indentation measurements were performed using the MFP-3D Atomic Force Microscope (Asylum Research, CA, USA). The hydrogels were maintained in saline after formation to ensure their hydrated state. The stiffness of each alginate gel was measured at n = 3 random locations and approximately 16 force curves were taken over a 4x4 grid at each location on each sample.

Diffusivity was examined by first forming alginate capsules using 10, 20, and 100 mM BaCl₂ without cells. 1 ml of alginate was used to form capsules for each BaCl₂ concentrations, and was loaded with 2 mg of bovine serum albumin (BSA, (Sigma-Aldrich)). The capsules were suspended in 2 ml of 0.9% saline, and the supernatant was sampled for released BSA over 24

hours. BSA was measured using the BCA total protein assay (Thermo Scientific) according to manufacturer's instruction, and analyzed using a Synergy 2 multi-mode Microplate Reader (BioTek, Winooski, VT, USA).

2.4. Differentiation of encapsulated hESCs

The stage-wise induction protocol for the pancreatic differentiation of hESCs was identical to our previous study, ending at the pancreatic progenitor stage instead of the maturation stage [4].

First, DE was induced using 100 ng/ml ActivinA (R&D Systems, Minneapolis, MIN) with 25 ng/ml Wnt3A (R&D Systems) for 4 days. Afterwards, PP was induced with 0.2 μ M KAAD-cyclopamine (CYC) (Millipore) for 2 days and 0.2 μ M CYC with 2 μ M retinoic acid (Sigma-Aldrich) for 2 days. All differentiation media was made using DMEM/F12 (Life Technologies), supplemented with 0.2% BSA and 1xB27® (Life Technologies).

2.5. Cell viability

LIVE/DEAD (Life Technologies) viability assay was performed according to manufacturer's instructions (details in SI methods).

2.6. Image analysis

The stock LIVE images from LIVE/DEAD analysis were processed using Metamorph. Briefly, the processing steps included thresholding to eliminate background, after which the image was binarized to create a black and white mask; filtered to detect the edges of the individual colonies, forming a mask or outline. From the mask, using Integrated Morphometry Analysis, data was generated for each colony within each image, measuring the area of the colony. This image processing was done on images taken on 5 capsules, each day for each condition.

2.7. hESC proliferation and death

Cell proliferation was measured using AlamarBlue (Life Technologies) assay according to manufacturer's instructions. Cell death was measured by analysis of released lactate dehydrogenase (LDH), using the CytoTox 96® Non-Radioactive Cytotoxicity assay according to manufactures instruction. Assay details are found in the SI methods.

2.8. qRT-PCR for gene expression analysis

Cells were decapsulated with 100 mM EDTA (Sigma) and washed twice with PBS before lysis. mRNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel, Bethlehem, PA). cDNA was obtained using ImpromII Reverse Transcription (Promega, Madison, WI). Each PCR reaction contained 5 μ l SYBR Green Master Mix (Agilent, Santa Clara, CA), 2 μ l nuclease free H₂O, 2 μ l primer, and 1 μ l cDNA. Samples were normalized to the house keeping gene GAPDH and analyzed relative to UD hESCs using the $\Delta\Delta$ Ct method. Gene expression was measured with quantitative polymerase chain reaction (qRT-PCR) using an MX3005P system (Agilent).

2.9. MagPix for TGF β pathway signaling analysis

The TGF β pathway was analyzed using the MILLIPLEX MAP TGF β Signaling Pathway Magnetic Bead 6-Plex (Millipore) for pSMAD2, pSMAD3, pERK1/2, and pAKT as well as total TGF β II and SMAD4 according to manufactures instruction (details in SI methods). Fluorescence intensity was measured using the xMAP (Luminex, Austin, TX) machine. The total protein was measured using a BCA total protein kit, according to manufactures instruction.

2.10. Immunostaining

Encapsulated cells were fixed with 4% formaldehyde for 20 min and cryopreserved with 30% sucrose for 24 hours before embedding in OCT for cryo-sectioning. H&E staining was done to

determine the gross morphology of the colonies for each encapsulation condition. For immunostaining, blocking was done with 10% donkey serum in PBS for 1 hour. Primary antibody staining was done overnight at 4°C, followed by addition of the secondary antibody for 45 minutes at room temperature. Finally, slides were covered with hardening mounting medium containing DAPI (Vectashield, Vector laboratory). Slides were washed three times with PBS in between each step of the staining protocol. Primary and secondary antibodies and dilution details are located in the SI methods.

2.11. Statistical analysis

Data were presented as mean $\pm$ SD. Statistical significance between two groups was determined using the two tailed Student T-test for two group comparisons. Statistical significance comparing multiple groups was determined using one-way ANOVA, with Tukeys or Games-Howell post hoc testing for homogeneous or inhomogeneous variance, respectively. Probability values at $P < 0.05(*)$ and $P < 0.01 (**)$ indicated statistical significance.

3.0. Results

3.1. Barium alginate stiffness, and not diffusion, affects encapsulated hESCs

The mechanical properties, namely stiffness, of organs can vary in a wide range: 1 – 10 kPa in soft tissue like brain or muscle, while hard tissue such as collagenous bone is on the order of 100 kPa [42]. Hence we first identified the range of cation concentrations which produces capsules in the range of 1 – 100 kPa, by varying barium concentrations (5 – 200 mM BaCl₂). Alginate capsules were formed by drop-wise addition of alginate into the cation solution, resulting in capsules of 2.07 ± 0.1 mm in diameter. Barium alginate capsules of this size have recently been shown to strongly resist fibrosis compared to traditionally used capsules of smaller diameter for up 175 days in immune competent mice, while supporting islet function and

viability [32]. Resulting capsule stiffness was measured using atomic force microscopy (AFM) micro-indentation to determine the Young's Modulus. Capsules created with less than 10 mM BaCl_2 did not form homogenous capsules (data not shown), while those created with greater than 100 mM BaCl_2 were outside the desired stiffness range noted above. The Young's modulus of the BAIG capsules progressively increased from 3.9 ± 1.3 kPA in the lowest BaCl_2 concentration (10 mM), to 73.3 ± 22.4 kPA in the highest BaCl_2 concentration (100 mM, Figure 1A).

The chosen range of capsule stiffness was next explored for encapsulating undifferentiated (UD) hESCs. Cells were suspended in 1.1% alginate with 0.2% gelatin and added to a solution of the desired BaCl_2 concentration for crosslinking (Schematic in Figure 1B). After 6 days of propagation, colony formation was minimal in the 50 and 100 mM BAIG capsules, as compared to cells encapsulated in 10 – 20 mM BAIG capsules (colonies indicated with white arrows, Figure 1C). Since the density of crosslinking could also possibly modulate the diffusivity of the capsule to nutrients, growth factors, or small molecules in the media, we measured BSA diffusivity with changing alginate crosslinking. BSA was chosen for this study for its high molecular weight of 66.5 kDA, in comparison to key differentiation inducers: ActivinA (26 kDA), Wnt3A (40 kDA), CYC (0.4 kDA), and Retinoic Acid (0.3 kDA). Figure 1D shows that there was insignificant difference in the release profiles of the BSA loaded BAIG capsules of different crosslinking densities after 24 hours. This indicates that for the range of BAIG used in this study, the encapsulated cells are unlikely to experience any difference in media exposure from diffusion limitations. On the other hand, the effect of crosslinking on the resultant capsule stiffness was significant.

3.2. Capsule stiffness suppresses the growth dynamics of encapsulated hESCs

We next quantified the effect of varying BaCl_2 concentration, and thereby BAAlg capsule stiffness, on the viability and proliferation of encapsulated hESCs throughout differentiation. UD hESCs were encapsulated using 10, 15, 20, 50, and 100 mM BaCl_2 , propagated for 6 days, at which stage they form visible colonies. Following this, encapsulated cells were induced to differentiate to DE and subsequently to PP, following the protocol detailed in Figure 2A [4, 6, 23]. Cell viability was evaluated on days 1, 6, 10, and 14 after encapsulation using the LIVE/DEAD assay. As illustrated in Figure 2B, all the gel conditions show similar viable cells (green fluorescence) on Day 1 after BAAlg encapsulation. However, by Day 6 after encapsulation, visible differences were apparent in the number of viable cells across each substrate condition. Softer capsules with lower BaCl_2 concentration exhibited larger viable cell colonies and less apoptotic single cells (red fluorescence). In contrast, stiffer BAAlg capsules synthesized with higher BaCl_2 concentration showed visibly fewer viable cell colonies and more apoptotic single cells. This effect was more dramatic by the end of the PP stage on day 14, where capsules of 50 mM and 100 mM BaCl_2 conditions resulted in very small cell colonies. However, in some of the capsules under these conditions, the colonies grew rapidly in size with unusual morphology as early as day 10 of differentiation (indicated by white arrows, Figure 2B). Visible cracks were observed on the surface of these capsules, likely due to the non-compliance of the stiffer capsules, which results in cracking as the capsule swells (Supplementary Figure 1). It is thus likely, that cracks also formed within the BAAlg capsule, allowing for uninhibited growth.

To further characterize the dynamics of cell growth, the images from the LIVE/DEAD assay were quantified using Metamorph image analysis software. The colony area (μm^2) of each live colony was measured, and the dynamics of average colony area for each capsule configuration was quantified (Figure 2C). In general, for all crosslinking conditions, the cell colony size increased initially with propagation. The extent of the growth was, however, strongly dependent on the capsule property. For example, by day 6, cells encapsulated using 10 mM BaCl_2 had a

larger area as compared to the other conditions. Overall, increased BaCl_2 concentration resulted in a progressive decrease in cell colony size. This trend was apparent at the end of propagation stage and continued through the rest of the differentiation stages, with later stages showing an enhanced effect of substrate composition on colony size. An exception to this was the 100 mM BAAlg condition, which had minimal growth in cell colonies all through the propagation and differentiation stages. As noted before, the 50 and 100 mM BAAlg conditions showed some uninhibited growth, potentially from gel cracking, which are depicted as outliers (circled in black) in Figure 2C. These results clearly show that growth of encapsulated hPSCs is strongly determined by properties of encapsulating gel, with increased gel stiffness suppressing growth.

Consistent with colony sizes, quantification of cell proliferation by the AlamarBlue assay also showed a strong dependence on substrate stiffness. As shown in Figure 3A, cells encapsulated in softer capsules (10-20 mM BaCl_2) are highly proliferative, and resulted in distinct colonies within the capsule. However, stiffer substrates (50, 100 mM BaCl_2) were less supportive of proliferation and hence failed to give rise to cell colonies. Along with proliferation, cell death was measured using the LDH assay, to verify if changing capsule properties are having any toxic effect on the cells. As shown in Figure 3B, cell death was most predominant in Day 1, immediately after encapsulation. Beyond that, cell death is somewhat suppressed for most of the conditions, while the cells remain highly proliferative as judged by AlamarBlue assay. It is important to note that cell seeding for all conditions was 1×10^6 cells/ml, and the peak death observed was less than 2.5×10^5 on day 1 for the 10 mM BaCl_2 condition. While this trend is consistent across the softer gels, cells encapsulated in the stiffer gels remain somewhat static. They are neither proliferative, nor show significant cell death. Overall, the above results clearly show that BAAlg capsules in the range of 10 – 20 mM BaCl_2 (~4-7 kPa) are supportive of hESC proliferation and colony formation. While the stiffer gels were not necessarily toxic, they did not support sufficient colony growth to be a feasible option for encapsulation.

3.3. Increasing capsule stiffness supports DE differentiation, while suppressing PP differentiation

Having confirmed cell viability and colony formation in BAAlg capsules, we next evaluated the feasibility of differentiating the cells after encapsulation and growth. Encapsulated hESCs under each BAAlg conditions were induced to DE and subsequent PP stage of differentiation following the protocol detailed in Figure 2A. Differentiation efficiency was analyzed by gene and protein expression for both DE and subsequent pancreatic commitment. Figure 4A, B compares the efficiency of germ layer commitment across varying capsule stiffness, which shows a distinct biphasic response. Mesendodermal markers *EOMES* and *CER* both showed an initial increase in expression with increased capsule stiffness (Figure 4A), reaching a peak upregulation of ~3800-fold at 50 mM BaCl₂ for *EOMES* and ~6900-fold at 20 mM BaCl₂ for *CER*. Further increase in substrate stiffness, however, resulted in a reduction in gene expression. A similar biphasic trend was also observed for the DE markers *SOX17* and *FOXA2*, with peak upregulation of ~8400-fold at 50 mM BaCl₂ for *SOX17* and ~29500-fold at 20 mM BaCl₂ for *FOXA2* (Figure 4B). Hence BAAlg capsules crosslinked with 20–50 mM BaCl₂ (~7–22 kPa) exhibited the most significant upregulation of mesendoderm and DE markers. However it is worth noting that even the lowest observed gene expression at 10mM BaCl₂ is still substantial differentiation as judged by the strong upregulation of relevant genes. Further analysis of *SOX17* and *FOXA2* protein expression by immunostaining confirmed the strong presence of cell colonies with significant expression of these proteins in 10, 15, and 20 mM BaCl₂ (Figure 4C).

Having confirmed germ layer commitment, the next step was analysis of the effect of substrate stiffness on pancreatic commitment. We first looked at *HNF4α*, which is a marker of the primitive gut tube, and is expressed in pancreatic development between the DE and PP stages.

Interestingly, consistent with the DE makers, gene expression of *HNF4α* increased as BA1g capsule stiffness increased (Figure 5A), but lacked the biphasic nature and was highest under the stiffest condition tested: ~510-fold increase over UD hESCs at 100 mM BaCl₂. Figure 5B shows gene expression of *PTF1A*, a precursor to PDX1, which showed a similar increasing trend with BA1g capsule stiffness, from ~3-fold to ~3300-fold upregulation over UD hESCs. A crucial transcription factor for endocrine pancreatic development is *PDX1*, which is also necessary for β cell maturation. In contrast with the previous analysis, *PDX1* gene expression showed strong inverse relationship with BA1g capsule stiffness (Figure 5C). The highest *PDX1* expression was obtained at 10 mM BaCl₂ which resulted in ~3800-fold increase over UD hESCs. All of the other gel conditions tested had a significantly lower *PDX1* gene expression (~370 – 580 fold increase over UD hESCs).

We subsequently compared the morphology of PP colonies across the gel conditions. At the end of PP induction, the BA1g capsules were dissolved and the collected cell colonies were sectioned and stained with H&E to visualize the colony morphology for each BA1g condition (Figure 5D). Cells differentiated in the 10 and 15 mM BA1g capsules resulted in colonies which were visibly less dense in higher BaCl₂ concentrations. The cell colonies within 10 and 15 mM BA1g were found to have distinct cavities (indicated with black arrows) within the colonies, which was significantly reduced at 20 mM condition and completely absent in 50 and 100 mM BA1g capsules. As indicated earlier, 50 and 100 mM BA1g capsules resulted in either small restricted colonies or in some cases, large elongated colonies.

Pancreatic differentiation was next confirmed by immunostaining of colony sections for PDX1. Consistent with the gene expression analysis, cells encapsulated in the 10 mM BaCl₂ gel were strongly positive for the protein PDX1 (Figure 5E). While PDX1 protein expression was also detected in 15 mM and 20 mM conditions, the number of PDX1 positive cells decreased

significantly with increasing gel stiffness. In the stiffest gels formed with 50 or 100 mM BaCl₂, no detectable cells were positive for the protein PDX1. Hence, while higher stiffness was conducive for mesendoderm and DE differentiation, the lower stiffness BA1g capsule was more supportive of endocrine pancreatic differentiation, as shown by high PDX1 gene and protein expression.

Since ECM can play a significant role in modulating cell fate, we further analyzed the PP colonies across gel conditions for relevant ECM proteins. In particular, laminin and collagen 1 are known to be important basement membrane and structural proteins in the pancreas, respectively [52-54] and were analyzed by immunostaining. Consistent with the differentiation data, the presence of ECM was more evident in lower stiffness gels (Figure 5F). Interestingly, the ECM deposition appeared to be localized primarily around the cavities within the colony (indicated with white arrows).

3.4. Capsule Properties impact signaling at both the DE and PP stage

Our results showed that in spite of identical chemical induction, the differentiation potential of the encapsulated cells was significantly modified by the properties of the encapsulating substrate. Further, the effect of the substrate was specific to the stage of differentiation and not consistent throughout. Since signaling pathways are also known to have similar stage specific effect on differentiation, we hypothesized that substrate properties are modulating the efficacy of relevant signaling molecules. The TGF β pathway is known to play a critical role in the process of endoderm and pancreatic differentiation. High activation is necessary for endoderm induction, while it is detrimental to pancreatic differentiation. Hence to analyze how substrate properties are affecting differentiation, we chose to measure the SMAD molecules, which are key effectors of the TGF β pathway. However, the complex interactions between parallel pathways make it difficult to understand pathway behavior by analyzing molecules and pathways in isolation [55].

Hence we also measured pERK and pAKT which are known to have significant interaction with the TGF β pathway. Using a Luminex-based MagPix assay, we measured the expression of relevant signaling molecules after the DE and PP stage in 10 and 20 mM BaCl₂ condition (Figures 6, 7). These conditions were chosen because they had the most contrasting effect between the DE and PP stage differentiation. We first focused on how gel stiffness influenced signaling at the DE stage (Figure 6A). Total TGF β RII levels were significantly lower in the 20 mM BAAlg, while t-SMAD4 levels were insensitive to changes in response to capsule stiffness. Interestingly, the primary effectors of the TGF β pathway, pSMAD2/3, showed an opposite trend. While pSMAD2 was significantly lower in the 20 mM BAAlg compared to 10 mM BAAlg, pSMAD3 was significantly higher. The cross talk molecule pERK1/2 also showed significantly higher expression in the 20 mM BAAlg, while pAKT, a negative regulator of the TGF β pathway, showed significantly lower expression in this condition compared to 10 mM BAAlg. To evaluate the level of negative regulation, we analyzed the ratio of pSMAD2/3/pAKT to estimate the level of available pSMAD2/3 which can influence differentiation (Figure 6B). While the ratio of pSMAD2/pAKT was not significantly different between the gel conditions, pSMAD3/pAKT was significantly higher in 20 mM BAAlg. Both higher levels of pSMAD3 and higher pSMAD3/pAKT ratio in 20 mM BAAlg condition, as compared to the 10 mM BAAlg condition, indicates a larger pool of active pSMAD3 molecules are available to direct mesendoderm gene transcription.

High TGF β signaling activation is of paramount importance for DE induction; however, following DE specification, activation of this pathway is detrimental to pancreatic commitment [56]. In our experimental set-up, beyond the DE stage there was no exogenous activation of the TGF β pathway. Even then we observed significant dynamics of the pathway molecules, the nature of which depended on the substrate properties (Figure 7A). Both 10 and 20 mM BAAlg conditions showed a significant increase in the receptor level and t-SMAD4 levels from DE to PP. However for 20 mM BAAlg, the key signaling molecules pSMAD2/3 remained similar between DE and PP.

This was surprising since the DE differentiation is conducted under high TGF β stimulation and the PP stage is devoid of it. Even more surprising was the response of the 10 mM BAIG condition. While pSMAD2 was significantly reduced from the DE to PP stage, as expected from the removal of ActivinA, pSMAD3 was significantly enhanced from DE to PP under the 10 mM BAIG condition. Furthermore, this increase in pSMAD3 is in disagreement with the enhancement of the PP stage differentiation we observe under the 10 mM condition, since SMAD molecules are known to interfere with PP induction [57]. pSMAD3 levels under 10 mM BAIG at the PP stage is not only increasing from the corresponding DE stage, it is also higher than the 20 mM BAIG at the PP stage. However, as mentioned earlier, it is difficult to conclude on the state of signaling by evaluating signaling pathways in isolation. Hence we also evaluated pAKT and pERK levels to characterize the signaling events in more detail (Figure 7A). We observed that both pERK and pAKT increased significantly from the DE to PP stage under both gel conditions. However, the difference was insignificant for both signaling molecules between the two conditions at the PP stage. These pathways alone do not explain the observed difference in differentiation. Hence we analyzed the ratio between the key signaling molecules, in order to quantify the interaction. We observed that the pSMAD2/3/pAKT ratio was significantly suppressed when the cells were differentiated from DE to PP, in both the gel conditions **with the exception of pSMAD2/pAKT for 20 mM BAIG** (Figure 7B). This indicates that the availability of active pSMAD levels lower upon withdrawal of ActivinA, while moving from DE to PP. While this analysis explains the feasibility of PP differentiation, it does not explain the superior performance of the 10mM BAIG condition.

We next specifically analyzed pathways which are being directly modulated for pancreatic induction. This includes inhibition of the sonic hedgehog (SHH) signaling pathway, the presence of which promotes liver bud formation [56]. Hence, we examined the substrate-specific effect of SHH inhibition by measuring the gene expression levels of the *SHH* ligand and *GLI1*, a

transcription factor downstream of and activated by SHH. Comparing the two gel conditions at the PP stage, we observed that cells encapsulated in 10 mM BAAlg capsules had lower expression of *SHH* and significantly lower expression of *GLI1*, compared to the 20 mM BAAlg condition (Figure 8A). However, for both of these conditions, the gene expression of *GLI1*, was significantly higher at the PP stage than the corresponding DE stage, even when the signaling was suppressed with the inhibitor CYC (Figure 8A). A previous report has shown that the TGF β pathway can activate SHH signaling through pSMAD3, independent of the SHH receptor [58]. Since we observed an increase in TGF β signaling activation at the DE stage in the stiffer 20 mM BAAlg conditions, we next wanted to examine if increased stiffness is activating SHH through the TGF β pathway. We inhibited TGF β signaling with the addition of Alk5i during the PP stage for the 20 mM BAAlg condition. However, Figure 8B shows that in the presence of the TGF β inhibitor, *PDX1* expression decreased, while *SHH* and *GLI1* expression increased slightly. This indicates that the stiffer substrates are more strongly activating SHH pathway independent of TGF β , and thus is detrimental to PP induction, even though these same conditions favored DE differentiation.

4.0. Discussion

The objective of this study was to evaluate the feasibility of using BAAlg encapsulation for pancreatic differentiation of hESCs, and how capsule properties regulate growth and differentiation. These capsules can then be subsequently used for implantation for T1D treatment, where BAAlg is expected to immunoisolate the cells within. For hPSC-derived β -cell therapy for T1D to reach fruition, high capacity biomanufacturing techniques are required to generate the necessary cell numbers ($\sim 10^9$ /patient). Hence, essential features to consider while engineering a platform for regenerative cell therapy for T1D are (i) supportive of cell growth (ii) supporting/promoting differentiation and (iii) amenable to immune protection, which has not yet

been demonstrated. We observed that DE differentiation was enhanced as capsule stiffness increased; however, high capsule stiffness was not supportive of cell growth and proliferation. Low stiffness capsules were supportive of both cell growth, and pancreatic differentiation. Signaling pathways analysis showed that increasing capsule stiffness activated TGF β signaling, which supports DE differentiation, and SHH signaling, suppressing PP differentiation. Hence in the current study we report for the first time, specific barium alginate configurations most supportive of growth and pancreatic differentiation of encapsulated hPSCs.

In evaluating the growth and differentiation properties of barium alginate capsules, the parameter we modulated was the crosslinking concentration. Even though the encapsulated cells were continuously induced by specific growth factors and chemical inducers, we hypothesized that the substrate properties play a critical role in modulating cell fate, as has been well known in stem cell literature, [40, 42, 59]. In islet encapsulation studies, 20 mM BaCl₂ capsules are the most ubiquitous, which results in a stiffness of 6.8 kPa. This is in a similar range as has been reported in mouse, 1.210 $\pm$ 0.77 [43], and adult human pancreata, 1.4 $\pm$ 2.1 kPa – 4.4 $\pm$ 5.1 kPa [60]. Based on this, we selected a wide range of cation concentrations encompassing this range, where capsules made using 10 – 20 mM BaCl₂ (~4 – 7 kPa) most closely mimicked the stiffness of the pancreas *in vivo*. As seen in previous reports, increasing BaCl₂ concentration increases the young's modulus, E (kPa), of the resulting alginate capsule [28, 48], which significantly affected both growth and differentiation of the encapsulated hESCs. Most significantly, BAAlg capsules using 10-20 mM BaCl₂ (first regime) was very supportive of cell proliferation, resulting in large viable cell colonies by the end of the 14 day differentiation protocol. In contrast 50 - 100 mM BaCl₂ (second regime) resulted in marginal proliferation into small colonies. Although gel stiffness increased as BaCl₂ concentration increased in the first regime, it was not adverse to cell growth and differentiation. While in the second regime, the resulting stress from the capsule stiffness was too high to sustain healthy cell growth. It was not

entirely clear if this poor viability was due to potential effects of barium toxicity [61], or from the high physical stress imparted by the capsule stiffness preventing cell proliferation. However, 1 day after hESC encapsulation, the highest cell death by the LDH assay was found in the lower barium concentration, which still showed 97% viability. Additionally, the high stiffness capsules were found to be cracking on the capsule surface and likely within the capsules, resulting in distinctly large and irregularly shaped cell colonies. This leads us to believe that the capsule was physically restraining the cells, and relieving the stress resulted in rapid cell growth in the cracked regions. The possibility of healthy cell growth in high barium capsule indicates the effects of barium toxicity in this system are minimal. This is further supported by the observation that better viability was observed in 100 mM BAlg, compared to the 50 mM BAlg on day 14 in Figure 2B. Barium concentration was higher in 100 mM BAlg, but cracking of the gel resulted in cell overgrowth, and more viable cells than in 50 mM BAlg, containing less barium. However, barium may still be toxic to the body in future implantation studies. Matching the most supportive capsule stiffness found in this study using a combination of calcium and low barium concentration, as done previously for islet encapsulation [62], may be useful in mitigating potential barium toxicity in the body. Taken together, this indicates that within the stiffness range of approximately 4 – 7 kPa, it is possible to maintain healthy growth and high expansion of hESCs, while beyond this range is not amenable to cell growth.

While the effect of stiffness was clear on cell viability and expansion, the effect of stiffness on pancreatic differentiation was much more complex. We evaluated the most supportive capsule properties to obtain PP cells, since implantation of PP cells have been widely reported to be successful in reinstating glucose homeostasis in diabetic mice upon implantation [63-66]. While the higher stiffness capsules are much stiffer than most soft tissue, it was not obvious how stiffness would influence differentiation in the presence of chemical differentiation factors. Remarkably, during both the DE and PP stage, alginate stiffness significantly influenced

differentiation, even though the soluble chemical cues were identical and there were no diffusion limitations. Even minimal change in stiffness (1.7 fold) between 10 and 20 mM BaCl₂ significantly affected differentiation. Similarly, Wilson *et al.* have shown that endoderm differentiation of alginate encapsulated mESC aggregates can be significantly increased by a minimal decrease in capsules stiffness (1.4 – 1.7 fold) [67]. In our study, both DE and early pancreatic differentiation had a positive correlation with BA_{lg} stiffness, showing increased stiffness enhanced differentiation. However, mesendoderm and endoderm differentiation had a slight bi-phasic behavior, with reduced differentiation in the high range, which was not observed in the pancreatic progenitor markers. The effect of stiffness on the key pancreatic endocrine transcription factor PDX1 was in complete contrast to the early markers. Increased gel stiffness strongly suppressed PDX1 expression, with the highest expression being at 10mM BaCl₂ (3.9 kPa). While this is completely opposite to the early pancreatic markers tested, an explanation can be that increased higher elastic moduli is suppressing or delaying differentiation and maintaining a progenitor pool. This is supported by the increase in gene expression with increased stiffness of *HNF4α*, a primitive gut tube marker involved in early pancreatic development, and *PTF1A*, a precursor to PDX1. Narayanan *et al.* have shown an approximately 10% increase in insulin and *PDX1* gene expression in hESC after pancreatic differentiation on 2D hyaluronic acid gels of stiffness 2.1 kPa compared to controls [68]. While this study only investigated a narrow stiffness range with only three gel stiffness's (1.3, 2.1, and 3.5 kPa) in 2D, it confirms that a delicate balance must be struck between physical and chemical cues.

The inconsistent effect of capsules stiffness on differentiation at the DE and PP stage is clearly indicative of the complex interactions between biophysical properties and signaling pathways. The mechanism of such interactions is still lacking in the context of stem cell differentiation, while understanding such interactions will significantly enhance the efficiency of engineering substrates for hPSC differentiation. To better understand such interactions, we evaluated the

expression of key molecules of the TGF β pathway, as well as key cross talk molecules. TGF β is a critical signaling pathway in hPSCs; however, its effects are specific to the stage of differentiation. Low level TGF β signaling is required for maintenance of pluripotency and self-renewal of hESCs, and high TGF β signaling induces DE differentiation [55]. While high levels are needed for DE differentiation, as mentioned previously, continued activation of TGF β is detrimental to further pancreatic differentiation. From our analysis of the effect of capsule stiffness on SMAD molecules, it is possible that the increased substrate stiffness is promoting DE stage differentiation by increased TGF β signaling activation. Previous work with UD hESCs [69, 70] and chondrocytes [71] have shown changes in substrate mechanical properties can result in TGF β signaling activation. Thus it is clear that physical stimuli imparted on the encapsulated cells can influence differentiation by modulating the signaling pathways. In analyzing the 10 and 20 mM BA1g conditions we observed a decrease in pSMAD2 levels in 20 mM from 10 mM BA1g, with a corresponding increase in pSMAD3 expression. However, since there is significant cross talk between pathways, parallel signaling pathways must also be examined. Specifically the PI3K pathway acts as a negative regulator of the TGF β pathway through sequestration of the R-SMADs by pAKT. Hence we measured the ratio of R-SMADs/pAKT as an estimate of R-SMADs available for gene transcription. While pSMAD2/pAKT was not significantly different, pSMAD3/pAKT was significantly higher in the 20 mM BA1g conditions, indicating higher TGF β activity by less negative regulation by the PI3K pathway. This trend follows previous reports for prostatic epithelial cells and human embryonic kidney cells, which have shown that pAKT suppresses TGF β signaling specifically through suppression of SMAD3 phosphorylation, and not SMAD2 [72, 73]. Additionally, we also examined pERK1/2 expression, a primary effector of the MAPK pathway. We observed significantly higher pERK1/2 expression in the 20 mM BA1g conditions. DE stage differentiation is induced by activation of the WNT pathway with Wnt3A, in addition to the TGF β pathways. Singh *et al.* have shown that pERK promotes DE stage differentiation by inhibition of the WNT

pathway inhibitor, Gsk3 β [55]. This indicates that the increase in DE stage differentiation as substrates stiffness increased may be due to higher TGF β and WNT signaling through a decrease in pAKT and GSK3 β , respectively.

While it is clear that increased substrate stiffness supports DE stage differentiation, the effect of stiffness on PP stage is more variable. Previous reports have shown that beyond DE commitment, TGF β signaling must be suppressed for subsequent pancreatic induction [56, 74, 75]. However analysis of the PP cells for signaling molecules revealed that even after removal of the TGF β ligand (Activin) and further PP induction, the TGF β pathway still remained active in a non-intuitive substrate dependent manner. The ratios of both pSMAD2/3/pAKT decreased from the DE to PP stage, indicating reduced levels of active pSMAD2/3 after the removal of ActivinA at the PP stage. However, this does not explain the superior PP stage differentiation at lower capsule stiffness. Hence we investigated the direct signaling pathways inducing PP differentiation; namely, retinoid signaling activation and sonic hedgehog (SHH) inhibition. Lopez-Carballo *et al.* have shown that retinoic acid treatment of human neuroblastoma cells activated PI3K signaling and resulted in increased AKT phosphorylation [76]. Induction of PP stage by addition of retinoic acid may serve to suppress TGF β signaling by the PI3K pathway, in addition to retinoid signaling activation. Inhibition of SHH signaling is achieved by addition of CYC, which inhibits SHH through the receptor smoothed [77]. Even in the presence of the CYC, we saw an increase in *SHH* ligand and its downstream transcription factor *GLI1*, after PP stage differentiation in 20 mM BAlg, as compared to 10 mM BAlg. Dennler *et al.* showed that even in the presence of CYC, TGF β induced expression of *GLI1* and *GLI2* specifically through SMAD3, in multiple human cell types [58]. Importantly, this activation of SHH signaling by TGF β was independent of the SHH receptor. Our results clearly show an increase in TGF β signaling in response to increased substrates stiffness at the DE stage. However, upon inhibition of TGF β at the PP stage with Alk5i in the 20 mM BAlg condition, we did not observe a rescue of *PDX1*. It is

thus unlikely that the increase in SHH in 20 mM BAAlg is TGF β mediated. This indicates the possibility that higher capsule stiffness is strongly increasing SHH signaling to the point where CYC cannot fully inhibit SHH, which has not been previously demonstrated. Abnormal hedgehog (HH) signaling is common in my human cancers, and can involve “non-classical” HH signaling activation independent of the “classical” ligand-smoothened HH activation [78]. As we have seen in our differentiation studies; this results in the ineffectiveness of HH signaling inhibitors like CYC. It is possible that the increase in substrate stiffness is activating “non-classical” SHH signaling, independent of the ligand-receptor binding.

5.0. Conclusions

In the present study we evaluated the feasibility of adopting BAAlg capsules as a unifying platform for hPSC growth, differentiation and implantation. Since BAAlg is well studied for islet transplantation, we focused our study on its usefulness for hPSC encapsulation and pancreatic differentiation. Our results clearly indicate the excellent performance of BAAlg capsules in supporting growth and pancreatic differentiation of encapsulated hPSCs, highlighting its potential as a single platform for hPSC scale-up, differentiation and final implantation. However, it also highlights the importance of considering capsule synthesis as an important parameter. Cell growth and differentiation were strongly dependent on the stiffness of the substrate. We determined that capsule stiffness of approximately 4 to 7 kPa was most supportive of cell proliferation, while ~3.9 was best for pancreatic differentiation. Analysis of the signaling pathway indicated increased stiffness strengthens TGF β signaling, driving DE differentiation. However, it also strengthens SHH signaling, which is detrimental to PP differentiation. Future implantation studies will first require investigation of the perm-selectivity of the barium alginate capsules with changing stiffness to determine the exact capsule parameters which support viability, differentiation, and immunoisolation from the patient immune system.

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Figure Legends

Figure 1: Characterization of Barium alginate capsule. A) Alginate hydrogel elastic moduli (Pa) as measured by AFM nano-indentation of alginate crosslinked with 10, 15, 20, 50, and 100 mM BaCl₂. B) Schematic of alginate encapsulation of hESCs with the desired BaCl₂ concentration. C) Brightfield images of hESCs encapsulated in alginate crosslinked with 10, 15, 20, 50, and 100 mM BaCl₂ after propagation of undifferentiated hESCs for 6 days to allow colony formation. White arrows indicate colonies, which were minimal in the 50 and 100 mM barium alginate conditions. Scale Bar is 450 μ m. D) BSA released from alginate capsules crosslinked with 10, 20, and 100 mM BaCl₂ over 24 hours. The results were considered significant if $P < 0.05$.

Figure 2: Viability and cell growth dynamics of encapsulated hESCs throughout differentiation. A) Stage-wise differentiation protocol for differentiation of hESCs to pancreatic progenitor (PP) cells. B) LIVE/DEAD assay of hESCs indicating cell viability in response to barium alginate capsule stiffness, on the first and last day of each stage of the differentiation protocol. Scale bar is 450 μ m. C) The stock LIVE images from LIVE/DEAD analysis were processed using Metamorph for each encapsulation condition on days 1, 6, 10, and 14 of the differentiation protocol. Using Integrated Morphometry Analysis, data was generated for each colony within each image, measuring the area of the colony.

Figure 3: Proliferation and cell death of encapsulated hESCs, in response to substrate stiffness. A) Cell proliferation measured by AlamarBlue assay, of encapsulated hESCs under each barium alginate

condition during pancreatic differentiation. B) Cell death measured by the LDH assay, of encapsulated hESCs under each barium alginate condition during pancreatic differentiation.

Figure 4: Increased substrate stiffness enhances DE stage differentiation. A) Relative gene expression of the mesendodermal genes *EOMES* and *CER* for each barium alginate condition at the DE stage, relative to undifferentiated hESC. B) Relative gene expression of the definitive endoderm genes *SOX17* and *FOXA2* for each barium alginate condition at the DE stage, relative to undifferentiated hESCs. C) Immunostaining of hESCs encapsulated with 10, 15, and 20 mM BaCl₂ at the DE stage, for *SOX17* and *FOXA2*. Immunostaining of hESCs encapsulated using 50 and 100 BaCl₂ was not performed due to the low yield of cells after DE differentiation for these conditions. Scale bar is 75 μ m. The results were considered significant if *P<0.05, **P<0.01.

Figure 5: Increased substrate stiffness suppressed PP stage differentiation. Relative gene expression of A) *HNF4 α* , B) *PTF1A*, and C) *PDX1* for each BAAlg condition, at the PP stage. Gene expression was normalized to undifferentiated hESCs. The results were considered significant if *P<0.05, **P<0.01. D) H&E staining of hESC colonies that were encapsulated using each cation concentration, after differentiation to the PP stage. Black arrows indicate cavities within cell aggregates, which were primarily seen in colonies encapsulation in 10 and 15 mM barium alginate capsules. Scale bar is 100 μ m. E) Immunostaining for *PDX1* of hESC encapsulated with 10, 15, 20, 50, and 100 mM BaCl₂ at the PP stage. Scale bar is 50 μ m. F) Immunostaining for the extracellular matrix proteins collagen 1 and laminin secreted by the cells within the colony, at the end of the PP stage. Scale bar is 75 μ m.

Figure 6: Effect of substrate stiffness on TGF β signaling at the DE stage. A) Expression of key signaling proteins of the TGF β pathway, and key cross talk pathway molecules by Magpix analysis, after DE stage differentiation for hESCs encapsulated using 10 or 20 mM BaCl₂. B) The ratios of pSMAD2/pAKT and pSMAD3/pAKT, which indicated the level of available pSMAD2/3. The results were considered significant if *P<0.05, **P<0.01.

Figure 7: Effect of substrate stiffness on TGF β signaling at the PP stage. A) Expression of key signaling proteins of the TGF β pathway, and key cross talk pathway molecules by Magpix analysis, after PP stage differentiation for hESCs encapsulated using 10 or 20 mM BaCl₂. B) The ratios of pSMAD2/pAKT and pSMAD3/pAKT, which indicated the level of available pSMAD2/3. The results were considered significant if *P<0.05, **P<0.01.

Figure 8: The effect of substrate stiffness on Sonic Hedgehog signaling. A) Gene expression of *SHH* ligand, and its downstream target *GLI1* at the DE and PP stage for hESCs encapsulated with 10 or 20 mM BaCl₂. Gene expression was normalized to undifferentiated hESCs. B) Gene express of *PDX1*, *SHH*, *GLI1*, at the PP stage for hESCs encapsulated using 20 mM BaCl₂, with the PP stage media supplemented with the TGF β receptor inhibitor Alk5i. Gene expression was normalized to hESCs encapsulated with 20 mM BaCl₂ at the PP stage, which where differentiated without the Akl5 inhibitor. The results were considered significant if *P<0.05.

graphical abstract

FIGURES

Figure 1

A

B

C

D

Figure 2

A

B

C

Figure 3

A

B

Figure 4

Figure 5

A

B

C

D

E

F

Figure 6

A

B

Figure 7

A

B

Figure 8

A

B